Outer Membrane Permeability and β-Lactamase Stability of Dipolar Ionic Cephalosporins Containing Methoxyimino Substituents

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Some enteric bacteria, such as *Enterobacter cloacae*, can develop high-level resistance to broad-spectrum cephalosporins by overproducing their chromosomally encoded type I β-lactamases. This is because these agents are hydrolyzed rapidly at pharmacologically relevant, low (0.1 to 1 μM), concentrations, owing to their high affinity for type I enzymes. In contrast, the more recently developed cephalosporins, with quaternary-nitrogen-containing substituents at the 3 position, show increased efficacy against β-lactamase-overproducing strains and, indeed, have a much lower affinity for type I enzymes. However, the possible contribution of an improved outer membrane permeability in their increased efficacy has not been studied. We found by proteoliposome swelling assays that cefepime, cefpirome, and E-1040 all penetrated the porin channels of *Escherichia coli* and *E. cloacae* much more rapidly than did ceftazidime and at least as rapidly as did cefotaxime. Considering that the influx of anionic compounds such as cefotaxime and ceftazidime will be further retarded in intact cells, owing to the Donnan potential, we expect that the newer compounds will penetrate intact cells 2 to 10 times more rapidly than will cefotaxime and ceftazidime. The kinetic parameters of hydrolysis of these agents by *E. cloacae* β-lactamase showed that at 0.1 μM, they were hydrolyzed much more slowly than was cefotaxime and at about the same rate as or a lower rate than was ceftazidime. The combination of these two effects explains nearly quantitatively why these newer agents are more effective against some of the β-lactamase-overproducing gram-negative bacteria.

Conventional assays showed that many of the more recently developed cephalosporins, such as cefoxitin, cefuroxime, and the broad-spectrum cephalosporins, including cefotaxime and ceftazidime, were totally resistant to hydrolysis by commonly encountered β-lactamases, including the type I or class C chromosomally encoded β-lactamases of members of the family *Enterobacteriaceae* (for an example, see reference 18). Nevertheless, the widespread use of these agents was followed by the emergence of resistant mutant strains of organisms such as *Enterobacter cloacae*, *Serratia marcescens*, and *Pseudomonas aeruginosa* which constitutively produced high levels of type I β-lactamases (15). This puzzling finding led to the proposal that the resistance was due to nonhydrolytic substrate binding by β-lactamase molecules (15, 20, 21, 24).

Vu and Nikaido (22) concluded, however, from quantitative considerations of outer membrane permeability and the number of β-lactamase molecules present in the cell, that such a nonhydrolytic trapping mechanism cannot explain the high level of resistance found in *E. cloacae* strains overproducing the type I β-lactamase. Furthermore, Livermore (8) and Vu and Nikaido (22) pointed out that conventional β-lactam hydrolysis assays with very high (0.1 to 10 mM) substrate concentrations may give misleading results because what is relevant for antibiotic activity is hydrolysis of the drug occurring at low (10 μM or lower) concentrations, at which the targets, i.e., the penicillin-binding proteins (PBPs), begin to become inhibited. Indeed, at such low concentrations, type I β-lactamases, which have a very high affinity or a low K_m for most broad-spectrum cephalosporins and cefoxitin, were shown to hydrolyze these "nonhydrolyzable" compounds at rates not much different from the rates of hydrolysis of the classical "hydrolyzable" compounds such as cefazolin (22). Thus, there is no need to assume a resistance mechanism based on nonhydrolytic binding or trapping (9, 10), and this was found to be true even with one of the most slowly hydrolyzed compounds, moxalactam, and with a bacterium with one of the least permeable outer membranes, *P. aeruginosa* (3).

Recently, several new cephalosporin compounds with an increased efficacy against β-lactamase overproducers were developed. In agreement with the notion that low hydrolysis rates at low substrate concentrations represent the most important factor in producing truly β-lactamase-stable compounds, they share low affinities or high K_m values for type I enzymes and are therefore expected to be hydrolyzed much more slowly in pharmacologically relevant situations. These compounds include cefepime (BMY-28142) (14), cefpirome (HR-810) (7), and E-1040 (23). They are characterized by containing, at the 3 position, substituents with quaternary nitrogen atoms (Fig. 1). Although it has been documented that type I enzymes hydrolyze these cephalosporins more slowly than the earlier compounds at low substrate concentrations (4, 7, 14), the other potential factor that contributes to the higher efficacy, increased penetration of the outer membrane (12), has not been studied. In this study, we examined these three cephalosporins in terms of their outer membrane penetration rates and their resistance to β-lactamases to gain a quantitative understanding of the increased efficacy of these compounds.

MATERIALS AND METHODS

Bacterial strains. *E. cloacae* 55 (wild type) and its mutant 55M, which constitutively produces the chromosomally encoded class C β-lactamase (2), were obtained from Christine Sanders. *P. aeruginosa* PAO1 was from the laboratory stock collection. Its derivative PAO4095 (FP⁻ *met-9020 pro-9024 blaI9407*), which constitutively produces the type I β-lactamase, was obtained from H. Matsumoto. For purification of TEM-1 β-lactamase, an *Escherichia coli* K-12 strain contain-
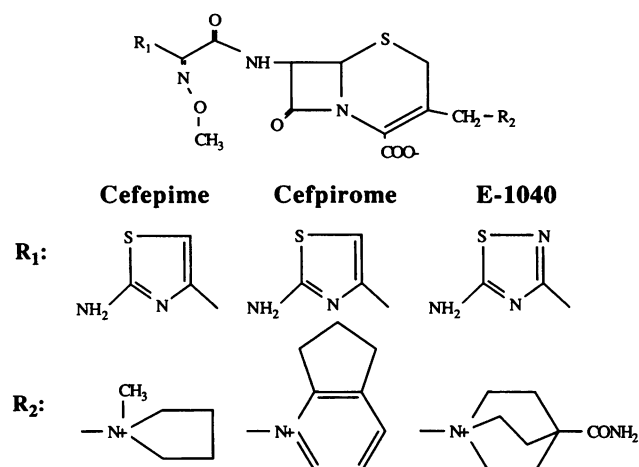
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FIG. 1. Structures of the dipolar ionic methoxyiminocephalosporins.

FIG. 2. Time course of hydrolysis of cefotaxime by the type I enzyme from *E. cloacae*. Partially purified enzyme (20 μg of protein) was added to 10 μM cefotaxime in 5 mM Tris chloride buffer (pH 7.5), and hydrolysis was monitored by measuring the optical density (O.D.) at 260 nm. Shown is an actual trace of the record on an expanded scale. At the arrow, the rate decreased to one-half the maximal rate (as ascertained by plotting of the first derivative), and the difference between the optical density at this point and the final optical density (represented by the vertical line) indicates that the substrate concentration at this point, i.e., the K_m, was 0.05 μM.

ing plasmid R_{417a} (12) was used. Other *E. coli* strains were described previously (12). All strains were grown in L broth (1% tryptone [Difco Laboratories], 1% yeast extract [Difco], and 0.5% NaCl) at 37°C.

Purification of porins. The *E. coli* OmpF porin was prepared as described earlier (13). Some properties of *E. cloacae* porins were reported earlier (17), but the strain used turned out to be *Klebsiella pneumoniae* (16). We recently found that *E. cloacae* 55 produced three porin species and purified each of the homotrimeric species by chromatography on a MonoQ (Pharmacia) column (W. Liu, S. Oya, and H. Nikaido, manuscript in preparation), a technique that was developed for the purification of homotrimers and heterotrimers of *E. coli* porins (1). For cephalosporin penetration studies, we used one species, called F, which produces the largest channel and whose synthesis is regulated in a way similar to that of the *E. coli* OmpF porin in being repressed in high-osmolarity media (Liu et al., in preparation).

Preparation of crude outer membrane. *P. aeruginosa* cells were suspended in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–NaOH buffer (pH 7.5) containing 0.5 mM phenylmethylsulfonyl fluoride and were broken by sonication in an MSE Soniprep 150 sonicator by four 30-s bursts interspersed with 30-s cooling periods. The mixture was centrifuged at 3,000 rpm in a Sorvall SS-34 rotor for 10 min to remove unbroken cells, and the supernatant was centrifuged at 12,000 rpm in the same rotor for 15 min. The pellet contained largely fragments of the outer membrane, which sediment faster because of their larger size and higher density (H. Nikaido, unpublished results).

Determination of outer membrane permeability. Rates of penetration of cephalosporins into porin channels were determined by the osmotic swelling of proteoliposomes reconstituted with purified porins or with outer membrane fragments. The method is similar to that described earlier (26), except that the cephalosporin solutions were adjusted to identical osmotic activity by the use of a vapor pressure osmometer (Wesco). When all compounds examined were dipolar ionic, for example, in the comparison of the newer cephalosporins with cephaloridine, the proteoliposomes were suspended in a 15% (wt/vol) solution of dextran T-40 (Pharmacia) containing 5 mM Tris chloride (pH 7.5) rather than the NAD-stachyose-imidazole buffer solution specified earlier (26), because a higher sensitivity was obtained by
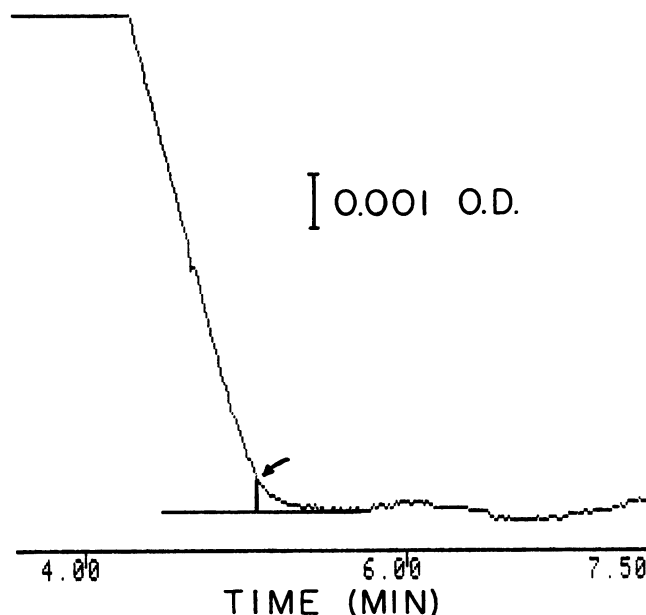
trapping the high-refractive-index dextran solution in the intravesicular space.

The ability of readily hydrolyzable cephalosporins to permeate intact cells was measured by coupling their diffusional influx across the outer membrane with their hydrolysis by the periplasmic β-lactamase (13). With *E. cloacae*, the permeability coefficients obtained tended to be higher with cells containing higher levels of β-lactamase, suggesting that some enzyme molecules may be present on the cell surface (3). Because of this problem, permeability was determined with the uninduced wild-type strain 55, but even these values should be considered maximal estimates.

Rates of hydrolysis by β-lactamases. The TEM-1 enzyme, the type I enzyme from *E. cloacae* 55M, and the type I enzyme from *P. aeruginosa* PAO4095 were purified essentially as described earlier (12), except that for *P. aeruginosa* cold $MgCl_2$ shock (5) was used instead of osmotic shock and the final MonoQ chromatography step was omitted.

The hydrolysis of cephalosporins was monitored in 5 mM Tris chloride buffer (pH 7.5) at 260 nm with a Lambda 4B spectrophotometer (The Perkin-Elmer Corp.). This spectrophotometer has extremely low noise (10^{-4} absorbance units at 0 absorbance unit) and drift, so that even the hydrolysis of compounds with very low V_{max} values could be monitored to completion (Fig. 2). When the K_m values were low, they could be obtained directly from a time course of the hydrolysis data. When the K_m values were moderately high (0.5 mM $> K_m > 0.1$ mM), an initial substrate concentration of 1 to 2 mM had to be used, and the problem of very high absorbance was overcome with a cuvette that had a 1-mm light path. For compounds with even higher K_m values, initial rates of hydrolysis were determined at various sub-

TABLE 1. Rates of permeation of cephalosporins through porin channels

Cephalosporin	E. coli OmpF porin		E. cloacae F porin		P. aeruginosa outer membrane	
	Relative permeation rate in liposomes[a]	Permeability coefficient of cells[b] (nm/s)	Relative permeation rate in liposomes[a]	Permeability coefficient of cells[b,c] (nm/s)	Relative permeation rate in liposomes[a]	Permeability coefficient of cells[b] (nm/s)
Cephaloridine	100	3,570	100	86	100	11
Cefepime	21	750	18	15	45	5
Cefpirome	18	643	16	14	10	1
E-1040	13	464	13	11	16	2
Cefotaxime	13	175	15	5	ND[d]	
Ceftazidime	7	95	4	1	ND[d]	

[a] Averages of several experiments.

[b] Obtained by normalizing the proteoliposome permeation rate with the permeability coefficient of intact cells of E. coli (13), E. cloaecae (this study), and P. aeruginosa (25) with cephaloridine. For compounds with a net negative charge of 1, correction for the effect of the Donnan potential, a factor of 2.66 (12), was applied.

[c] Because of the possibility of some fraction of the enzyme being present on the cell surface, these values are maximal estimates, and the true permeability may be even lower.

[d] It was not possible to determine the rates of penetration of anionic and dipolar ionic compounds with a single proteoliposome preparation (see the text).

strate concentrations, and Lineweaver-Burk plots were used.

The total β-lactamase activity per unit weight of cells was determined with cephaloridine and crude sonic extracts of the cells (12).

MIC determination. MICs were determined with twofold serial dilutions in L broth and Mueller-Hinton broth. Approximately 10^4 cells were inoculated into 0.5 ml, and the results were read after 24 h. Little difference was seen between the two media used.

RESULTS

Rates of penetration of cephalosporins into porin-containing proteoliposomes. Proteoliposomes were reconstituted with purified porin proteins from E. coli and E. cloacae or with crude outer membrane fragments from P. aeruginosa. For the latter organism, total outer membrane rather than purified individual proteins was used because there is currently a controversy over the identity of its porin molecule (for a review, see reference 11). They were diluted in iso-osmotic solutions of the cephalosporins, and the relative permeation rates were calculated from the initial rates of swelling of the proteoliposomes (Table 1). The newer cephalosporins penetrated the porin channels of E. coli and E. cloacae at rates about equal to or somewhat higher than the rate for cefotaxime and much more rapidly than did ceftazidime. The implications of these data in terms of permeability of intact cells are presented in the Discussion.

Kinetics of enzymatic hydrolysis. Enzymatic hydrolysis was determined spectrophotometrically (Table 2). The data for cefepime were similar to those of Hiraoka et al. (4), who reported a K_m of 260 μM and a V_{max} which was 0.04% that of cephalothin, a value corresponding to 0.008% that of cephaloridine, if we use the conversion factor obtained with the enzyme from E. cloacae 55M. Although the relative V_{max} was higher in our study, this difference was most likely due to differences in assay conditions or in the enzymes produced by different E. cloacae strains.

Although the V_{max} values were significant with the TEM-1 enzyme, all of the newer compounds showed very high K_m values with this enzyme, so that the rates of hydrolysis at pharmacologically relevant low concentrations (22) were negligible.

MICs. To evaluate the contributions of outer membrane penetration rate and β-lactamase stability to the efficacy of various agents, we determined MICs with strains producing type I β-lactamases at different levels (Table 3). The newer compounds were usually more efficacious than were the broad-spectrum cephalosporins in β-lactamase-overproducing strains. Thus, in E. coli, the overproduction of the enzyme did not increase the MICs of the newer cephalosporins at all. Whereas the MICs for the overproducing E. cloacae strains were usually over 128 μg/ml with cefotaxime, values in the range of 0.2 to 2 μg/ml have been reported with the newer compounds (7, 14, 23). We obtained somewhat higher MICs for the latter compounds, in the range of

TABLE 2. Kinetics of enzymatic hydrolysis of cephalosporins

Cephalosporin	Type I enzyme						TEM-1 enzyme		
	E. cloacae			P. aeruginosa					
	K_m (μM)	V_{max}[a]	$v(0.1 μM)$[b] (%)	K_m (μM)	V_{max}[a]	$v(0.1 μM)$[b] (%)	K_m (μM)	V_{max}[a]	$v(0.1 μM)$[b] (%)
Cephaloridine	170	100	100	35	100	100	350	100	100
Cefepime	80	0.05	0.11	87	0.05	0.02	5,000	2.4	0.17
Cefpirome	130	0.16	0.2	38	0.18	0.17	4,000	3.7	0.33
E-1040	300	0.02	0.01	400	0.05	0.001	3,000	0.8	0.09
Cefotaxime	0.05	0.086	97	0.03	0.02	5.4	9,500	2.4	0.09
Ceftazidime	2.5	0.006	0.4	3	0.002	0.02	>30,000	—[c]	0.0003

[a] Relative value, calculated by normalizing to the V_{max} value with cephaloridine.

[b] Calculated relative rate of hydrolysis at a substrate concentration of 0.1 μM.

[c] —, The V_{max} is unknown because the K_m is too high. However, the rate at a 1 mM substrate concentration is known (12).

TABLE 3. MICs for strains producing various levels of the type I enzyme

Strain	Enzyme activity[a]	MIC (μg/ml) in L broth				
		Cefotax-ime	Ceftaz-idime	Cefep-ime	Cef-pirome	E-1040
E. coli						
LA5	0.096[b]	0.03	0.5	0.13	0.13	0.13
LA51	0.23[b]	0.5	2	0.13	0.13	0.13
TE18	1[b]	4[c]	16[c]	0.13	0.13	0.13
E. cloacae						
55	0.06	0.25	0.25	0.13	0.13	0.13
55M	39	>256	256	8	16	8
P. aeruginosa						
PAO1	0.09	16	2	1	4	0.25
PAO4095	0.57	256	8	4	16	0.25

[a] V_{max} (micromoles per milligram of protein per minute) of sonic extract with cephaloridine as the substrate.

[b] From Nikaido and Normark (12).

[c] These values are much higher than those reported by Nikaido and Normark (12). Most probably this is because in the present study a high-osmolarity medium was used, resulting in the repression of the wide porin channel, OmpF, and the lowering of permeability for cephalosporins.

8 to 16 μg/ml for *E. cloacae* 55M, but these MICs are still much lower than the cefotaxime MIC of >256 μg/ml and the ceftazidime MIC of >128 μg/ml. Similarly, although the cefotaxime MIC increased from 16 μg/ml in wild-type *P. aeruginosa* (strain PAO1) to 256 μg/ml in the β-lactamase-constitutive mutant (strain PAO4095), the MICs of the newer agents remained fairly low. These data are analyzed in the Discussion.

DISCUSSION

Cephalosporin permeation rates in intact cells. We measured the rates of penetration of dipolar ionic cephalosporins into proteoliposomes (Table 1). Since the permeability coefficients of intact cells for another dipolar ionic cephalosporin, cephaloridine, could be measured with the Zimmermann-Rosselet assay (13), we were able to normalize the data for other dipolar ionic cephalosporins to those for cephaloridine and calculate the permeability coefficients shown in Table 1. Compounds such as cefotaxime or ceftazidime, which carry a net negative charge, are further retarded by the interior negative Donnan potential in penetrating intact cells (19). Therefore, a correction factor had to be applied in converting penetration rates for proteoliposomes, which have no Donnan potential, to permeation rates for intact cells (Table 1, footnote *b*).

Although in proteoliposome assays the newer cephalosporins showed penetration rates only marginally faster than that of cefotaxime, their penetration of intact cells is predicted to be much more rapid than is that of the representatives of the broad-spectrum compounds, cefotaxime and ceftazidime (Table 1), primarily because the dipolar ionic compounds are not retarded by the Donnan potential (19). The newer compounds, cefepime, cefpirome, and E-1040, contained the methoxyimino moiety on the α-carbon of the 7 substituent, a structural feature that was found to decrease the rates of penetration of monoanionic cephalosporins into the *E. coli* OmpF porin by as much as a factor of 10 (26). In comparison with cephaloridine, the newer compounds also had much slower rates of penetration, but perhaps the decrease was less than that in the monoanionic compounds,

especially when we consider the large sizes of some of the newer compounds. It was noted earlier (26) that the hydrophobicity, a solute parameter which had a strong negative influence on the rate of diffusion of anionic cephalosporins through the porin channel, had much less of an influence on the permeation rates of dipolar ionic cephalosporins. The present data further suggest that other steric features of the molecule also may have less of an effect on the penetration rates if the solutes are dipolar ionic.

Thus, all of the three newer compounds tested are expected to penetrate *E. coli* and *E. cloacae* several times faster than are cefotaxime and ceftazidime (Table 1). Unfortunately, comparisons of this type were not possible with *P. aeruginosa* because the large channel size of the porin(s) of this organism allowed the diffusion of NAD$^+$ and stachyose, precluding the use of these compounds for the suspension of proteoliposomes.

Affinity of the agents for β-lactamases. It is clear that the goal of producing cephalosporins with a low affinity for type I or class C β-lactamases has been achieved with all of the newer compounds (see the K_m values listed in Table 2). When the hydrolysis rates at a 0.1 μM substrate concentration were calculated on the basis of these data, all of the newer cephalosporins were hydrolyzed at least 30 times more slowly than was cefotaxime by *E. cloacae* and *P. aeruginosa* enzymes (Table 2). There was less of a difference between ceftazidime and the newer compounds, but the latter compounds were still hydrolyzed at least twice as slowly as was ceftazidime by the *E. cloacae* enzyme (Table 2). However, with the *P. aeruginosa* enzyme, the rates of hydrolysis of cefepime and cefpirome were about equal to and faster than that of ceftazidime, respectively. In contrast, E-1040 showed exceptional stability against this enzyme (Table 2).

Contributions of increased permeability and increased enzyme stability to the efficacy of the newer cephalosporins. Three factors determine the efficacy of β-lactams against gram-negative bacteria (12). These are (i) outer membrane permeability, (ii) stability against periplasmic β-lactamases, and (iii) affinity for the target. Clearly, the newer cephalosporins are much more effective against β-lactamase-over-producing strains, as indicated by the MICs determined in this study (Table 3). Although quantitative studies of affinity for PBPs do not appear to have been published, we shall now discuss the potential contribution of outer membrane permeability and β-lactamase stability in more quantitative terms.

Permeability and stability enhance efficacy by increasing the probability that the drug will reach the target. This probability is proportional to the target access index (TAI) (12), as follows:

$$TAI = PA[V_{max}/(K_m + C_{inh})]^{-1} \qquad (1)$$

where P, A, and C_{inh} denote the permeability coefficient of the outer membrane, the area of the outer membrane per unit weight of cells, and the lowest concentration of the drug needed to inhibit the most sensitive of the essential PBPs. V_{max} and K_m denote the kinetic constants of the periplasmic β-lactamase. The $P \times A$ portion of the equation obviously reflects the rate of penetration of the outer membrane, and the $V_{max}/(K_m + C_{inh})^{-1}$ portion indicates the probability of the drug successfully negotiating the β-lactamase barrier. If we assume that the newer agents have an affinity for PBPs that is not much different from those of cefotaxime and ceftazidime (C_{inh} estimated to be approximately 0.1 μM), then the β-lactamase stability term of equation 1 can be

thought of as the reciprocal of the hydrolysis rate at a 0.1 μM substrate concentration (Table 2).

When the newer agents were compared with cefotaxime in *E. cloacae*, clearly the more than 400-fold increase in β-lactamase stability appeared to be more important than the increase in permeability, estimated to be two- to fourfold (Table 1). However, when they were compared with ceftazidime which, among the broad-spectrum cephalosporins, is exceptionally stable against type I enzymes, the significant (around 10-fold) increase in the calculated permeability was more important than the sometimes modest (twofold with cefpirome) increase in the stability of the newer compounds. Furthermore, in *P. aeruginosa*, cefpirome was much less stable than was ceftazidime against the chromosomally encoded enzyme (Table 2), yet it appeared to be as efficacious against the enzyme overproducer as was ceftazidime (both showing a fourfold increase in the MIC in comparison with that against the wild type; Table 3), a result strongly suggesting that an increased ability of cefpirome to permeate compensated for the decreased enzyme stability. Thus, although the lower affinity of the newer cephalosporins for class C β-lactamases has often received more attention, at least in some cases the improvement in efficacy appears to be due more to the improvement in permeability. We believe that this result emphasizes the importance of improving the ability of β-lactam agents to permeate the outer membrane. Even slowly penetrating agents may be quite effective when dealing with organisms with an inefficient β-lactamase barrier, but any improvement in permeability will increase efficacy by increasing the TAI (see equation 1). This ability will become especially important when efficacy becomes marginal because of the presence of a strong β-lactamase barrier.

Is the improvement in efficacy explained by the changes in TAI? The TAI is related to the MIC, as follows:

$$\text{MIC} = C_{\text{inh}}(\text{TAI}^{-1} + 1) \qquad (2)$$

For our β-lactamase-constitutive strain of *E. cloacae*, 55M, which produced the type I enzyme at a specific activity of 33.3 μmol/mg per min with 1 mM cephaloridine, calculation showed that the TAIs for ceftazidime and cefotaxime were extremely low, 0.003 and 0.00003, respectively. Thus, TAI^{-1} will be much larger than 1, and the increase in the TAI will produce a nearly proportional decrease in the MIC. When we compared cefepime with ceftazidime, for example, there was an 11-fold improvement in the penetration rate and about a 3-fold improvement in β-lactamase stability. The maximal decrease in the MIC expected from these changes is 11 × 3, or 33-fold. Indeed, there was at least a 32-fold difference between the MICs of ceftazidime (256 μg/ml) and cefepime (8 μg/ml) for strain 55M.

Are there significant differences among the three newer agents studied? This is a complex issue, as the differences are sometimes very small and as we do not know an important parameter, i.e., the affinity for PBPs. It appears that cefepime penetrates slightly better than the other two (Table 1), most probably because of its smaller size and generally hydrophilic structure. In terms of stability against the type I enzymes tested, E-1040 appears to have a significant advantage. However, since the kinetic parameters of type I enzymes are notoriously sensitive to the assay conditions (6), we feel that one should excercise caution in drawing conclusions. In any case, it seems clear that all three compounds have a very pronounced advantage over the broad-spectrum cephalosporins when we consider the combined effect of outer membrane permeability and stability against class C enzymes under pharmacologically relevant conditions.

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